EFFECTS OF THE CALPAIN PROTEASES ON THE UBIQUITIN-PROTEASOME SYSTEM AND PROTEIN SYNTHESIS SIGNALING IN RAT SKELETAL MUSCLE

By

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Dedicated to my family

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Skeletal muscle atrophy can be a debilitating problem, and in extreme cases life threatening. Muscle wasting is a consequence of reduced protein synthesis and/or elevated protein degradation. While the ubiquitin-proteasome pathway (UPP) accounts for most of the elevated protein degradation, proteases acting upstream of the UPP may initiate the degradative process. Since the calpain proteases have been implicated in the initial events leading to elevated protein breakdown, one goal of the present investigation was to determine whether the calpain proteases act upstream of the UPP in skeletal muscle. Calpain activation had no discernible effect on the UPP (i.e., the level of myofibrillar/cytosolic ubiquitin-conjugated proteins or proteasome enzyme activity). However, the calpain-mediated rise in total protein degradation was prevented by proteasome inhibition, indicating that the proteasome is necessary for the processing of calpain cleavage products. In fact, acute calpain activation increased proteasome-dependent protein degra-

dation by 45%. Our data indicate that calpain acts upstream of the proteasome system; thus calpain may be an initial step in elevated protein degradation.

Akt plays a critical role in protein synthesis, and the molecular chaperone heat shock protein90 (HSP90) appears to regulate Akt activity. Since HSP90 is a calpain substrate, calpain activation may subsequently inactivate Akt and its immediate downstream elements. Calpain activation significantly reduced HSP90β content and Akt activity. Calpain also inhibited Akt's downstream component mammalian target of rapamycin (mTOR). While activated Akt inhibits glycogen synthase kinase-3β (GSK-3β), a negative regulator of protein synthesis, calpain activation increased GSK-3β activation.

Our data implicate calpain as an initial factor in elevated muscle proteolysis since these proteases appear to act upstream of the proteasome. The novel finding that calpain adversely affects the Akt/mTOR/GSK-3β signaling pathway suggests that calpain also contributes to reduced protein synthesis. Finally, given the multitude of functions carried out by molecular chaperones, the finding that calpain reduces the level HSP90β suggests that calpain activation may affect an assortment of cellular functions. Taken together, these data indicate that calpain likely plays a prominent role in the loss of muscle proteins during catabolic conditions and thus offers a practical target for intervention.

CHAPTER 1
INTRODUCTION

Skeletal muscle is a highly adaptive tissue and the adaptive response depends upon the stimuli. Bed rest, cast immobilization, and space flight reduce skeletal muscle use and cause deleterious alterations to skeletal muscle such as reductions in muscle protein content and profound muscle atrophy, and these modifications are more pronounced in slow-twitch muscles (Jaspers and Tischler, 1984; Desplanches et al. 1987). Skeletal muscle atrophy has important implications because the loss of muscle mass contributes to muscle weakness and reduced functional capacity. The maintenance of muscle mass requires a balance between the rates of protein synthesis and protein degradation, and it is well established that this equilibrium is altered during disuse (Jaspers and Tischler, 1984; Thomason et al. 1989). The rate of protein synthesis declines within hours of disuse, reaching a new suppressed steady state by the second day of disuse (Thomason et al. 1989). Proteolysis, on the other hand, begins to rise after two days of muscle disuse and reaches maximal activation at approximately the fifteenth day (Thomason et al. 1989). Scientists have long hypothesized that countermeasures normalizing either or both aspects of protein metabolism during muscle disuse would likely attenuate disuse muscle atrophy. Three major proteolytic systems have been identified in skeletal muscle: the lysosomal, calcium dependent, and ubiquitin-proteasome pathways. Because the lysosomal proteases do not degrade myofibrillar proteins (Wildenthal and Wakeland 1985; Lowell et al. 1986; Fernandez and Sainz, 1997), which represent approximately 70% of total

muscle protein, the contribution of the lysosomal proteases to muscle protein turnover is likely minimal (Mitch and Goldberg, 1996). In fact, the lysosomal proteases do not contribute significantly to elevated proteolysis observed in several catabolic conditions including cancer cachexia (Temparis et al. 1994), sepsis (Tiao et al. 1994), and reduced skeletal muscle use (Tischler et al. 1990; Taillandier et al. 1996). The ubiquitin-proteasome pathway, on the other hand, seems to be the primary proteolytic system in skeletal muscle. This system degrades the large majority of proteins under normal physiological conditions and in various catabolic states (Rock et al. 1994; Taillandier et al. 1996; Craiu et al. 1997; Tawa et al. 1997; Fang et al. 1998; Hobler et al. 1998). Proteins degraded by the ubiquitin-proteasome system are first marked for degradation by the attachment of ubiquitin to the target protein; this ubiquitin-conjugation leads to rapid degradation of the target protein by the proteasome (Grant et al. 1995; Mimnaugh et al. 1996; Thrower et al. 2000). The content of ubiquitin-conjugated proteins increases with reduced skeletal muscle use (Ikemoto et al. 2001), as does proteasome enzymatic activity (Shanely et al. 2002). Further, it is widely held that the ubiquitin-proteasome system is responsible for muscle atrophy in catabolic conditions because this system accounts for the large majority of elevated muscle proteolysis following reduced muscle use (Taillandier et al. 1996), skeletal muscle denervation (Tawa et al. 1997), sepsis (Hobler et al. 1998), cancer cachexia (Temparis et al. 1994), and burn injury (Fang et al. 1998). Clearly, the ubiquitin-proteasome proteolytic pathway has a key role in elevated proteolysis during muscle disuse. However, activation of the ubiquitin-proteasome system may be due to elevated substrates made available by another proteolytic pathway, the calcium activated proteolytic system.

Although the calcium activated proteases, the calpains, remain inactive under normal physiological conditions, these proteases have been implicated in muscle pathologies associated with the loss of calcium homeostasis, such as sepsis and Duchenne muscular dystrophy (Williams et al. 1999; Spencer and Mellgren, 2002). Recent research indicates that reduced skeletal muscle use causes a rise in cytosolic calcium (Ingalls et al. 2001), and it has further been demonstrated that reduced use causes activation of the calpain proteases (Taillandier et al. 1996; Shanely et al. 2002). Importantly, calpain inhibition has been shown to attenuate both accelerated proteolysis and skeletal muscle atrophy associated with reduced skeletal muscle use (Shanely et al. 2002; Tidball and Spencer, 2002). Although the ubiquitin-proteasome pathway and the calpain proteases play a role in disuse atrophy, evidence is accumulating for the hypothesis that the calpain proteases may be an initial step in ubiquitin-proteasome system activation. For example, while the proteasome does not degrade intact myofibrillar proteins (Koohmaraie, 1992; Solomon and Goldberg, 1996), several myofibrillar proteins are calpain substrates (Koohmaraie, 1992; Lim et al. 2004). Moreover, calpains cleave relatively few peptide bonds in a limited number of proteins (Goll et al. 1991; Azarian et al. 1995; Huang and Forsberg, 1998). Because the calpain proteases do not degrade proteins to small peptides or amino acids, it has been hypothesized that calpain cleavage products become substrates for the ubiquitin-proteasome system (Huang and Forsberg, 1998). Indeed, a number of mechanisms exist whereby calpain activation could lead to activation of the ubiquitin-proteasome pathway. The N-end rule pathway appears to be the primary ubiquitination pathway in skeletal muscle (Solomon et al. 1998b) and calpain cleaves proteins at amino acid residues that may promote ubiquitin-conjugation via the N-end rule pathway. Dam-

aged and misfolded proteins are also targeted for degradation by the ubiquitin-proteasome pathway (Hiller et al.1996; Ramanathan et al. 1999). Thus, calpain cleavage products may be recognized as such, and degraded by the ubiquitin-proteasome system.

Calpain proteolysis may also indirectly contribute to protein ubiquitination. Collectively, heat shock proteins (HSPs) are known as molecular chaperones because they properly fold newly synthesized proteins, refold damaged proteins, and transport proteins to various subcellular compartments (Beckmann et al. 1990; Kang et al. 1990; Jakob et al. 1993). These proteins play a critical role in normal cellular function and during the cellular response to stress. In non-skeletal muscle cells, heat shock protein 90 (HSP 90) is a calpain substrate and recent research indicates that calpain activation destabilizes the HSP 90/client protein complex (i.e., reduces the binding of HSP 90 to its client protein) (Stalker et al. 2003). The molecular complex formed between HSP 90 and its client proteins appears to be necessary to client protein stability because HSP 90 inhibition, which also destabilizes the HSP 90/client protein complex, causes degradation of numerous HSP 90 client proteins by the ubiquitin-proteasome pathway (Mimnaugh et al. 1996; Whitesell and Cook, 1996; An et al. 2000; Basso et al. 2002). Thus, calpain activation may indirectly promote ubiquitin-conjugation by preventing HSP 90 from performing its vital chaperone functions, subsequently leading to ubiquitin-conjugation and proteasome degradation of HSP 90 client proteins.

Hence, several lines of evidence suggest that caplain activation may initiate a cascade of events leading to ubiquitin-proteasome system activation. While the idea that the calpain and the ubiquitin-proteasome systems work in sequence has been proposed, the question has yet to be addressed experimentally.

Calpain activation may also play a role in reduced protein synthesis caused by skeletal muscle disuse. The insulin-like growth factor 1 (IGF-1) pathway intermediate Akt activates several downstream targets known to mediate protein synthesis (Oldfield et al. 1994; Kimball et al. 1998; Kawasome et al. 1998; Kimball et al. 1999) and previous investigations have convincingly demonstrated the key role of Akt signaling in regulating skeletal muscle size (Bodine et al. 2001b; Rommel et al. 2001; Reynolds et al. 2002). For example, the Akt signaling pathway is activated during skeletal muscle overload, and muscle disuse reduces Akt activation below control levels (Bodine et al. 2001b; Rommel et al. 2001). Further, skeletal muscle atrophy is attenuated in denervated skeletal muscles concomitantly treated with an active form of Akt (Bodine et al. 2001b). Importantly, recent studies indicate that Akt exists in a chaperone/client complex with HSP 90 (Sato et al. 2000; Takahashi and Mendelsohn, 2003). This association appears essential to proper Akt function and stability, since destabilization of the HSP 90/Akt complex causes inactivation of Akt, Akt degradation, and a reduction in Akt protein content (Sato et al. 2000; Basso et al. 2002; Solit et al. 2003; Xu et al. 2003; Fujiwara et al. 2004). As described above, HSP 90 is a calpain substrate and calpain activation destabilizes the HSP 90/client complex in non-skeletal muscle cells (Stalker et al. 2003). These data suggest that calpain activation may destabilize the HSP 90/and Akt complex, and consequently inactivate a signaling protein known to play a critical role in protein synthesis signaling and the maintenance of muscle mass, although this hypothesis has yet to be tested.

In summary, reduced-use skeletal muscle atrophy is a consequence of reduced protein synthesis and elevated protein degradation. The calpain proteolytic system and ubiquitin-proteasome proteolytic pathway are both activated during skeletal muscle dis-

use. It is well established that ubiquitin-conjugated proteins are degraded by the proteasome, and calpain cleavage may cause protein modifications that lead to ubiquitin-proteasome degradation. Although calpain activation may subsequently activate the ubiquitin-proteasome system, this hypothesis remains to be tested.

Research indicates that the Akt signaling pathway plays a key role in protein synthesis signaling and the trophic state skeletal muscle. The chaperone protein HSP 90 and Akt form a molecular complex, and this association positively modulates Akt activity and stability. HSP 90 is a calpain substrate and calpain activation has been shown to destabilize the HSP 90/client protein complex in non-skeletal muscle cells. Therefore, activation of the calpain proteases may reduce the binding of HSP 90 and Akt, and consequently reduce Akt signaling activity. If this is the case, then calpain activation may suppress the activation of a protein previously shown to mediate protein synthesis signaling and skeletal muscle mass. However, this question has yet to be addressed experimentally.

While calpain activation has been shown to destabilize the HSP 90/client protein complex in non-skeletal muscle cells, no data are available regarding the effects of calpain activation of HSP 90 chaperone function in skeletal muscle. The molecular complex formed between HSP 90 and its client proteins appears to play a central role in both ubiquitin-proteasome degradation and Akt signaling. Therefore, if calpain activation does in fact activate the ubiquitin-proteasome system and reduce Akt signaling, elucidating whether calpain activation also destabilizes the HSP 90/client protein complex in skeletal muscle may offer insight as to the potential mechanisms of these outcomes. The following questions were addressed in this study.

Questions

1. Do the calpain proteases act upstream of the ubiquitin-proteasome proteolytic pathway in rat skeletal muscle?

2. Does an increase in calpain proteolytic activity reduce in protein synthesis signaling in rat skeletal muscle?

3. Does calpain activation reduce the association of HSP 90 and Akt in rat skeletal muscle?

Hypotheses

1. The calpain proteases will act upstream of the ubiquitin-proteasome pathway in rat skeletal muscle.

2. Increased calpain proteolytic activity will reduce protein synthesis signaling in rat skeletal muscle.

3. Calpain activation will reduce HSP 90/Akt binding in rat skeletal muscle.

CHAPTER 2
REVIEW OF LITERATURE

Skeletal muscle is particularly sensitive to changes in its use. Bed rest, cast immobilization and space flight reduce skeletal muscle use and lead to significant muscle atrophy. Slow-twitch/antigravity muscles, such as the soleus, are more sensitive to reductions in use and this muscle can atrophy by 44% with 14 days of disuse (Koesterer et al. 2002). Affected muscles become weak and easily fatigable, consequently limiting functional capacity and increasing the risk of injury. Further, muscle wasting may necessitate months of rehabilitation to regain functional capacity. Delineating the mechanisms of reduced-use muscle atrophy is essential to developing countermeasures to prevent this type of muscle wasting.

Alterations in Protein Metabolism Caused by Reduced Skeletal Muscle Use

Skeletal muscle proteins, like all proteins, are constantly degraded and replaced by newly synthesized proteins. Consequently, the maintenance of muscle mass depends on the precise regulation of protein synthesis and protein degradation (Mitch and Goldberg 1996). This delicate balance becomes disrupted during periods of reduced muscle use, as the rate of protein synthesis declines and rate of proteolysis increases with disuse (Booth and Seider, 1979; Jaspers and Tischler, 1984; Thomason et al. 1989, Munoz et al. 1993). In the soleus muscle, the rate of protein synthesis decreases within hours of disuse and reaches a new reduced steady state by the second day of unloading (Thomason et al. 1989). Protein degradation, however, begins to rise after two days of unloading, reaching

maximum activation at about the fifteenth day of muscle unloading (Thomason et al. 1989). Three major proteolytic systems have been identified in skeletal muscle: the lysosomal, calcium-activated, and ubiquitin-proteasome pathways (Taillandier et al. 1996). Although each of these proteolytic systems becomes activated with muscle disuse, the contribution of the lysosomal proteases to muscle proteolysis is likely minimal since these proteases do not degrade myofibrillar proteins (Wildenthal and Wakeland, 1985; Lowell et al. 1986; Fernandez and Sainz, 1997), which account for approximately 70% of total muscle protein. Indeed, research indicates that the lysosomal proteases do not contribute significantly to elevated proteolysis observed in a number of catabolic conditions including cancer cachexia (Temparis et al. 1994), sepsis (Tiao et al. 1994), and reduced skeletal muscle use (Tischler et al. 1990; Taillandier et al. 1996). The ubiquitin-proteasome pathway, on the other hand, degrades the majority of intracellular proteins under normal conditions and there is evidence that this pathway may be responsible for the bulk of increased muscle proteolysis during disuse (Rock et al. 1994; Craiu et al. 1997; Solomon and Goldberg, 1996; Taillandier et al. 1996; Tawa et al. 1997; Ikemoto et al. 2001).

The Ubiquitin-Proteasome Proteolytic System and Reduced Muscle Use

The ubiquitin-proteasome system degrades short-lived normal proteins, long-lived normal proteins, and abnormal proteins (Hershko et al. 1982; Chau et al. 1989; Craiu et al. 1997; Rock et al. 1994). The system can be divided into two distinct and sequential phases: ubiquitination of the protein, thus targeting the protein for degradation, and degradation of polyubiquitinated proteins by the proteasome (Chau et al. 1989; Seufert and Jentsch, 1992). Protein ubiquitination requires the ubiquitin-activating enzyme (E1),

ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) (Hershko et al. 1983). In this process, E1 activates ubiquitin in an adenosine triphosphate (ATP) dependent manner, and transfers ubiquitin to E2. Next, E3 transfers ubiquitin from E2 to the target protein. This procedure continues, thus forming a polyubiquitin chain on the target protein. Once ubiquitinated by four or more ubiquitin molecules, the target protein is degraded by proteasome (Thrower et al. 2000).

Reduced skeletal muscle use has a profound impact on the ubiquitin proteasome system. First, muscle disuse is associated with an increase in the level of mRNA for several components of the ubiquitin-proteasome pathway (Taillandier et al. 1996; Ikemoto et al. 2001). Increases in the content of ubiquitin conjugated proteins (Ikemoto et al. 2001) and proteasome proteolytic activity also accompany reduced muscle use (Shanely et al. 2002). Importantly, *in vitro* experiments using specific protease inhibitors indicate that the ubiquitin-proteasome pathway accounts for the large majority (~70%) of the elevated muscle proteolysis observed during muscle disuse (Taillandier et al. 1996). For this reason, it has been widely held that the ubiquitin-proteasome pathway is responsible for elevated protein degradation caused by muscle disuse. However, there is evidence that the calcium-activated proteases may initiate the breakdown of muscle proteins, and subsequently activate the ubiquitin proteasome system. If the pathways are sequential, then the calcium-activated proteases may be an initial step in ubiquitin-proteasome system activation and, consequently, elevated proteolysis.

The Calcium-Activated Proteolytic System: General Background

The calcium-activated proteases, termed calpains, are classified as ubiquitous isoforms or tissue specific isoforms, based on their expression (Sorimachi et al. 1994). Al-

though a skeletal muscle specific calpain isoform has been identified (calpain III / p94), the proposed experiments will investigate the role of the ubiquitous calpains as this type has been shown to play an important role during reduced skeletal muscle use (Shanely et al. 2002; Tidball and Spencer, 2002). The two ubiquitous calpain isoforms have been termed micro-calpain and milli-calpain (μ-calpain and m-calpain, respectively). The calpain nomenclature reflects the calcium concentration required to induce their half-maximal proteolytic activation *in vitro*; μ-calpain and m-calpain require approximately 1-50 μM and .2-1 mM calcium concentrations for activation, respectively (Barrett et al. 1991; Edmunds et al. 1991). Because these calcium concentrations are higher than values generally observed under physiological conditions, endogenous co-activators that reduce the calcium necessary for calpain activation likely exist. Indeed, protein co-activators and phospholipids lower the calcium required for calpain activation *in vitro* (Michetti et al. 1991; Saido et al. 1992; Melloni et al. 1988). Autolysis, the removal of the N-terminal peptide tail of both subunits, has also been observed to reduce the calcium necessary for calpain activation (Suzuki et al.1981). In addition, a phosphorylation cascade may modulate calpain activation since calpain has been shown to be phosphorylated *in vitro* and *in vivo* (Kuo et al. 1994). While the mechanism(s) of calpain activation are still poorly understood, the finding that *in vivo* calpain inhibition has a protective effect indicates that the proteases are active under physiological conditions (Ray et al. 2001; Tidball and Spencer, 2002).

The ubiquitous calpains consist of a large catalytic subunit (~80 kDa) and a small regulatory subunit (~30 kDa) (Tsuji and Imahori, 1981). The subunits are divided into six domains; the large subunit contains domains I thru IV and the small subunit consists of

domains V and VI (Goll et al. 2003). The three residues that make up the catalytic core (also known as the catalytic triad) are situated in domains I and II (Alexa et al. 2004). The third domain contains calcium and phospholipid binding sites (Tompa et al. 2001). Because phospholipids reduce the calcium concentration required for calpain activation (Saido et al. 1992), it is thought that domain III may coordinate the lipid/calcium activation signal (Tompa et al. 2001). Domain III may also mediate the activation signal originating from domain IV to the protease domain, given its physical location between the primary calcium-binding domain (domain IV) and the proteolytic domain (Carafoli and Molinari, 1998). The fourth and sixth domains are very similar and each contains five calcium-binding sites (Hosfield et al. 1999). Crystal structure experiments of domain VI revealed that sites 1, 2, and 3 bind calcium at a much higher affinity than site 4, whereas site 5 does not bind calcium (Blanchard et al. 1997). The calcium binding sites likely play a critical role in calpain activation as simultaneous ablation of calcium binding at sites 1-3 in both subunits increases the calcium required for calpain activation from 325 μM to 7.4 mM (Dutt et al. 2000). The function of domain V is unknown at present; however, scientists speculate that this domain may serve as an anchor responsible for binding calpain to the cell membrane (Brandenburg et al. 2002).

The conformational change(s) necessary for calpain activation are still poorly understood. There have been several reports that the calpain subunits reversibly dissociate in the presence of calcium, and subunit dissociation has been correlated with calpain activation (Melloni et al. 1984; Yoshizawa et al. 1995; Kitagaki et al. 2000). However, Graham-Siegenthaler and coworkers (1994) demonstrated that the large subunit alone lacks catalytic activity, while the associated subunits exhibit full proteolytic activity. Impor-

tantly, other independent research groups have also observed a rapid loss of proteolytic activity in dissociated calpain (Nakagawa et al. 2001; Pal et al. 2001). In addition, it has been demonstrated that the subunits co-immunoprecipitate at a calcium concentration sufficient for calpain activation and during caplain proteolysis (Zhang and Melgren, 1996). Finally, in studies using immonofluorescence and confocal microscopy, it was determined that the two subunits remain associated following the activation of calpain in cells (Gil-Parrado et al. 2003). Based on these data, most, but not all, scientists agree that the calpain subunits remain associated under physiologically relevant conditions.

Recently, the crystal structure of calpain revealed that, in the absence of calcium, the catalytic triad residues contained in domains I and II are misaligned (Hosfield et al. 1999). Consequently, the active site is incapable of substrate hydrolysis. It was hypothesized that anchor peptides between domains I and VI, and domains II and III hold the catalytic triad residues apart in the absence of calcium, and that release of these constraints is required for calpain activation (Hosfield et al. 1999). However, in a subsequent study using a construct containing only domain I and II, it was determined that calcium binding at the proteolytic domains was sufficient to induce conformational changes in the catalytic domains and activate the enzyme (Moldoveanu et al. 2002). Based on the two experiments, the following calpain activation sequence was proposed by Moldoveanu et al. (2002): the binding of calcium to the binding sites in domains IV and VI induces conformational changes in domains III, IV and VI. These conformational changes lead to the release of contact between domains I and VI, and domains II and III (and possibly small unit dissociation). Autolytic cleavage of the N-terminal region of both subunits modulates calpain activation by lowering the calcium necessary for proteolytic activity. Finally, the

binding of calcium to domains I and II brings the catalytic triad residues into proper alignment to form a functional active site. While this model of activation is appealing, it is, nonetheless, speculative and further research will be required for a complete understanding of the conformational changes necessary for calpain activation.

Calpains Endogenous Inhibitor: Calpastatin

Calpastatin is the endogenous inhibitor of the calpains, and like the calpains, calpastatin is ubiquitously expressed (Takano et al. 1984). Calpastatin binds to and inhibits both μ-calpain and m-calpain in a calcium dependent manner (Cottin et al. 1981; Maki et al. 1988). While the signal initiating calpastatin activation is unknown, conformational changes in calpain may be the stimulus for calpain-calpastatin binding since there is no evidence that calpastatin binds calcium (Goll et al. 2003). Calpastatin contains four repeating inhibitory domains (domains 1-4), and each domain can independently inhibit calpain (Emori et al. 1987; Maki et al. 1987). Each domain possesses three subdomains designated A, B, and C (Goll et al. 2003). Region B of calpastatin binds calpain at (or near) the catalytic domain, thus acting as a competitive inhibitor (Maki et al. 1988). Subdomains A and C bind to calpain domains IV and VI, respectively, and in this way act to enhance the inhibitory efficiency of subdomain B (Emori et al. 1988; Ma et al. 1994; Takano et al. 1995). However, calpain degrades calpastatin in the presence of calcium, and this fragmentation reduces calpastatin activity (Nakamura et al. 1989; De Tullio et al. 2000). The currently accepted concept is that calpastatin serves as a "suicide substrate" for calpain (Nakamura et al. 1989) and the degradation of calpastatin by calpain liberates the protease thereby allowing continued calpain proteolysis.

Calcium Dysregulation and Calpain Activation in Skeletal Muscle

The physiological functions of the calpains in skeletal muscle are poorly understood. However, research suggests that the calpains have an important role in myoblast migration and fusion in developing muscle (Kumar et al. 1992; Temm-Grove et al. 1999; Dedieu et al. 2004). In mature muscle, the calpains have been implicated in muscle pathologies typified by loss of calcium homeostasis such as sepsis and muscular dystrophy (Williams et al. 1999; Spencer and Mellgren, 2002). Importantly, skeletal muscle disuse also leads to a disruption in calcium homeostasis. Using a popular model of reduced skeletal muscle use, hind limb unloading, it was determined that the cytosolic calcium concentration in soleus muscles increased by 38% following two days of unloading, and by day seven, cytosolic calcium reached 117% of control values (Ingalls et al. 2001). In addition, countermeasures designed to maintain calcium homeostasis during disuse have been shown to be beneficial, as the administration of calcium channel release blockers during muscle disuse attenuates the loss of myosin heavy chain, elevated proteolysis, and skeletal muscle atrophy (Tischler et al. 1990; Soares et al. 1993; Wagatsuma et al. 2002). Hence, disruptions in calcium homeostasis during reduced muscle use likely lead to activation of the calpain proteolytic system. Indeed, increases in the level of calpain mRNA and calpain proteolytic activity also accompany skeletal muscle disuse (Taillandier et al. 1996; Shanely et al. 2002), and calpain inhibition following a relatively acute exposure to reduced skeletal muscle use (18 hours of mechanical ventilation) attenuates the rise in total proteolysis (Shanely et al. 2002). Further, inhibition of the calpain proteases throughout the entire period of disuse attenuates skeletal muscle atrophy (Tidball and Spencer, 2002). Thus, the loss of calcium homeostasis and subsequent calpain activation

likely plays an important role in skeletal muscle disuse atrophy. In fact, these proteases may be an initial step in elevated muscle proteolysis.

Evidence for Sequential Proteolytic Systems: Calpain Cleavage Products and the Ubiquitin-Proteasome System

As mentioned above, the ubiquitin-proteasome pathway has also been implicated in skeletal muscle disuse atrophy. However, a large body of evidence suggests that the calpain proteases may act "upstream" of the ubiquitin-proteasome system, and if this is the case, then calpain activation may be the initial step in activation of the ubiquitin-proteasome system. Previous investigations indicate that the proteasome does not degrade intact myofibrillar proteins (Koohmaraie, 1992; Solomon and Goldberg, 1996), indicating that these proteins must first be released from the sarcomere by other proteases before being degraded by the proteasome. Despite differing in the calcium concentration required for their activation, μ-calpain and m-calpain have similar, if not identical, substrates (Croall and DeMartino, 1984; Ojho et al. 1999). While skeletal muscle contractile proteins such as myosin and actin are poor calpain substrates (Pemrick and Grebeneau, 1984; Goll et al. 1991), other sarcomeric proteins that are important to structural integrity are excellent calpain substrates. Titin and nebulin, two proteins that connect myofilaments to the Z-disk, are rapidly degraded by calpain (Huang and Forsberg, 1998; Lim et al. 2004). Indeed, a number of sarcomeric proteins have been identified as calpain substrates, including: tropomyosin, and troponins -T and -I (Koohmaraie, 1992; Reid et al. 1994; Delgado et al. 2001). Calpains also degrade the critical cytoskeletal proteins talin, desmin, dystrophin, spectrin, and vinculin (Nelson and Traub, 1983; Cottin et al. 1992; Yoshida et al. 1995; Koh and Tidball, 2000; Serrano and Devine, 2004). Moreover, incubation of skeletal muscle strips with calcium or purified calpain results in the complete

removal of Z-disks (Busch et al. 1972; Goll et al. 1991). Importantly, the calpain proteases cleave relatively few peptide bonds in their protein substrates, thereby generating large polypeptide fragments (Nelson and Traub, 1983; Cottin et al. 1992; Lim et al. 2004; Serrano and Devine, 2004). Given the limited proteolytic action of the calpain proteases, scientists speculate that the peptide fragments resulting from calpain cleavage become substrates for the ubiquitin-proteasome system (Huang and Forseberg, 1998; Goll et al. 2003). Calpain cleavage may indeed serve as a degradation signal, or degron, for the ubiquitin-proteasome system. As described previously, ubiquitin-conjugated proteins are degraded by the proteasome. A number of protein modifications can cause ubiquitin-conjugation. Proteins that are perceived as abnormal are ubiquitinated (Hershko et al. 1982), as are misfolded proteins (Hiller et al. 1996) and damaged proteins (Shang et al. 2001). Therefore, calpain cleavage products may be perceived as abnormal or damaged, ubiquitin-conjugated and degraded by the proteasome.

One well-described ubiquitin-conjugation pathway is the N-end rule pathway. The N-end rule relates the N-terminal amino acid residue of a protein to its half-life (Bachmair et al. 1986). Notably, the N-end rule pathway of protein ubiquitination is responsible for the majority of ubiquitin-conjugation in normal and atrophying skeletal muscle (Solomon et al. 1998a; Solomon et al. 1998b). In the N-end rule pathway, proteins with certain destabilizing N-terminal amino acid residues (in place of the usual N-terminal) are recognized, ubiquitin conjugated, and rapidly degraded by the proteasome (Bachmair et al. 1986; Gonda et al. 1989; Richter-Ruoff et al. 1992). Proteins bearing either bulky hydrophobic, basic, or acidic N-terminal amino acid residues serve as a degron for this pathway (Bachmair et al. 1986; Gonda et al. 1989). Importantly, calpains cleave at least

three amino acids meeting these criteria: tyrosine, which has some hydrophobic characteristics; methionine, which is characterized as hydrophobic; and arginine, which is both basic and hydrophobic (Sasaki et al. 1984). Further, Nelson and Traub (1983) demonstrated that calpain cleaves both desmin and vimentin at the N-terminal of the protein. Hence, calpain cleaved polypeptide fragments bearing destabilizing N-terminal residues may become substrates for the predominant ubiquitin conjugating system in skeletal muscle, the N-end rule pathway.

Calpain, HSP 90, and the Ubiquitin-Proteasome System

Calpain activation may also prevent other proteins from performing their physiological functions, and in this way, cause ubiquitin-proteasome degradation of proteins. Molecular chaperones are proteins that bind to and stabilize other proteins (Hartl, 1996). Chaperone proteins have a variety of cellular functions; they associate with the polypeptide chain on the ribosome and stabilize the translation complex, thus facilitating protein translation (Beckmann et al. 1990). These proteins also aid in the proper folding of newly synthesized proteins, and transport "client proteins" to various subcellular locations (Beckmann et al. 1990; Kang et al. 1990). In addition, molecular chaperones play an essential role in salvaging damaged proteins by refolding these proteins to their functional conformation (Lee and Vierling, 2000). Certain proteins require molecular chaperones for stability. For example, the molecular chaperone heat shock protein 90 (HSP 90) binds to its so-called client proteins, thus forming a chaperone/client complex (Sato et al. 2000; Basso et al. 2002). The chaperone/client interaction appears to be essential to client protein stability because HSP 90 inhibition, which destabilizes the chaperone/client protein complex (i.e. reduces the binding of HSP 90 with its client proteins), causes degradation

of numerous HSP 90 client proteins by the ubiquitin-proteasome system including: the glucocorticoid receptor, Akt, endothelial nitric-oxide synthase (eNOS), p53 and the pro-oncogenic proteins nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), $p185^{c\text{-}erbB\text{-}2}$, $p210^{bcr\text{-}abl}$, and v-scr (Whitesell and Cook, 1996; Mimnaugh et al. 1996; Whitesell et al. 1998; An et al. 2000; Basso et al. 2002; Jiang et al. 2003; Bonvini et al. 2002). Importantly, independent laboratories have demonstrated that HSP 90 is a calpain substrate in non-skeletal muscle cells (Minami et al. 1994; Bellocq et al. 1999; Su and Block, 2000). Moreover, recent research indicates that calpain proteolysis destabilizes the HSP 90/client complex in mesenteric tissue (Stalker et al. 2003). Therefore, if calpains cause HSP 90/client protein dissociation in skeletal muscle, as they do in non-skeletal muscle cells, it seems to reason that HSP 90 client proteins may also become substrates for the ubiquitin-proteasome pathway in skeletal muscle. If this is the case, then calpains may indirectly contribute to activation of the ubiquitin-proteasome system by prohibiting HSP 90 from performing its role as a molecular chaperone. Thus, calpain cleavage products may be recognized as abnormal proteins or by their destabilizing N-terminal residue and ubiquitin-conjugated, while destabilization of the HSP 90/client complex by calpain may cause ubiquitination of HSP 90 client proteins.

Contribution of the Calpains to Proteolysis: Rationale for Conflicting Findings

The contribution of the calpains in reduced-use skeletal muscle atrophy is a matter of debate. Overexpression of calpastatin, the endogenous calpain inhibitor, attenuates reduced-use skeletal muscle atrophy (Spencer and Tidball, 2002) and, as described above, calpain inhibition following a relatively brief period of reduced muscle use prevents accelerated proteolysis (Shanely et al. 2002). However, because calpain inhibition follow-

ing longer catabolic treatments such as, muscle denervation, fasting, metabolic acidosis, cancer cachexia, sepsis, and reduced muscle use (Furuno et al. 1990; Medina et al. 1991; Mitch et al. 1994; Baracos et al. 1995; Tiao et al. 1994; Taillandier et al. 1996) fails to suppress elevated muscle proteolysis, it is widely held that the calpain proteases do not contribute significantly to skeletal muscle atrophy in catabolic conditions. Importantly, the approach utilized to determine the contribution of the calpains to total proteolysis was similar in each of the aforementioned experiments, and this method may not accurately assess the role of the calpains. In these experiments, skeletal muscles were removed 3 to 10 days after induction of the catabolic treatment, and incubated in a physiological solution containing protease inhibitors to determine the contribution of the various proteolytic systems to protein degradation. For example, Taillandier and colleagues (1996) subjected rats to hind limb unloading for nine days. Following unloading, soleus muscles were removed and incubated in a physiological solution containing protease inhibitors to determine the relative contribution of the various proteases to muscle proteolysis. Using this model it was estimated that the ubiquitin-proteasome pathway accounted for approximately 70% of the elevation in total proteolysis, while the calpain (and lysosomal) proteases contributed about 18%. Importantly, the model described above fails to account for the potential contribution of the calpains to ubiquitinated proteins during the nine days of skeletal muscle disuse, since calpain inhibition was undertaken only after nine days of disuse. Namely, the content of ubiquitin conjugated proteins increases with disuse, and if calpain products become ubiquitinated, then the calpain proteases contribute to this increase in proteasome substrates. Further, given the build-up of proteasome substrate (ubiquitin-conjugated proteins) at the time of calpain inhibition, preventing further pro-

duction of substrates would likely have little immediate impact on elevated proteasome activity, and thus total proteolysis. If the two proteolytic pathways are sequential, then an acute catabolic treatment, in which there is not a large "back-up" of proteasome substrates, may reveal the contribution of the calpains to total muscle proteolysis via the ubiquitin-proteasome pathway. Hypothetically, the calpain products would move rapidly through the ubiquitin-proteasome system. This idea is supported by a recent study conducted by Du et al. (2004). In that study, the scientists treated L6 skeletal muscle cells with an active form of the apoptotic protease caspase-3 for two hours to determine whether the protease would break down the actomoysin protein complex. It was determined that the protease does, in fact, cleave actomyosin, and it was further demonstrated that the caspase-3 cleavage products were rapidly degraded by the ubiquitin-proteasome pathway. In addition, even limited cleavage of actomyosin by caspase-3 caused a large increase in ubiquitin-proteasome system mediated protein degradation. Thus, in the early catabolic phase, the ubiquitin-proteasome pathway may also be capable of rapidly degrading calpain cleavage products. However, in chronic conditions, in which there is an abundance of proteasome substrates, the contribution of the calpain proteases to total proteolysis may be masked. Determining whether that calpain proteases act upstream of the ubiquitin-proteasome system may explain the conflicting findings regarding the contribution of the calpains to total proteolysis. While it has been suggested that the calpain and ubiquitin-proteasome systems work in sequence, this hypothesis has not been addressed experimentally. If the pathways are sequential, then calpain may be an initial step in ubiquitin-proteasome system activation and consequently, elevated proteolysis.

Potential Role of the Calpains in Protein Synthesis Signaling

As mentioned previously, reduced-use skeletal muscle atrophy is thought to be the consequence of both a decline in protein synthesis and a rise in protein degradation. While the calpains have been implicated in elevated muscle proteolysis, these proteases may also contribute to the reduction in protein synthesis during skeletal muscle disuse. As mentioned above, the molecular chaperone HSP 90 forms a chaperone/client complex with the signaling protein Akt (Sato et al. 2000; Basso et al. 2002). This association positively modulates Akt activation, as inhibition of HSP 90 chaperone activity destabilizes the chaperone/client protein complex and significantly reduces Akt activity (Sato et al. 2000; Fujiwara et al. 2004). Furthermore, destabilization of the chaperone/client protein complex causes degradation of Akt, and a reduction in Akt protein content (Basso et al. 2002). Since calpain cleavage of HSP 90 destabilizes the chaperone/client complex (Stalker et al 2003), calpain cleavage may reduce the binding of HSP 90 and Akt, and consequently inactivate Akt. Importantly, Akt is a signaling intermediate in the insulin-like growth factor 1 (IGF-1) signaling pathway (Rommel et al. 2001), and this signaling pathway known to mediate protein synthesis (Bragado et al. 1998).

The Role of Akt in Protein Synthesis

Binding of IGF-1 to its receptor causes activation of phosphatidylinositol 3-kinase (PI3K) (Rommel et al. 2001). PI3K stimulation subsequently leads to phosphorylation of Akt, thereby activating Akt (Rommel et al. 2001). In turn, Akt activates at least two signaling pathways that mediate the translation phase of protein synthesis. Once activated, Akt phosphorylates and inactivates GSK3 (Rommel et al. 2001), a negative regulator of eukaroyotic initiation factor 2B (eIF2B). eIF2B plays an important role in the GDP/GTP

exchange reaction of eIF2. During translation initiation, eIF2 transfers initiator methionyl-tRNA (met-tRNA$_i$) to the 40S ribosomal subunit to form the 43S pre-initiation complex in a GTP dependent manner (Darnborough et al. 1973; Levin et al. 1973). Once eIF2 delivers met-tRNA$_i$, GTP is hydrolyzed and eIF2/GDP is released from the complex (Merrick, 1979). eIF2B then exchanges the eIF2/GDP for GTP and this GDP/GTP recycling is necessary for another round of initiation to begin (Konieczny et al. 1983; Williams et al. 2001).Thus, Akt phosphorylation inactivates GSK-3β (Rommel et al. 2001), thereby facilitating eIF2B GDP/GTP recycling.

Akt also activates the mammalian target of rapamycin (mTOR) (Rommel et al 2001). Downstream targets of mTOR include p70S6K and eIF4E binding protein (4E-BP) (Rommel et al. 2001). p70S6K activates ribosomal S6; S6 promotes selective synthesis of proteins involved in mRNA translation such as ribosomes (Kawasome et al. 1998). 4E-BP binds to eIF4E, thereby prohibiting eIF4E from performing its role in protein translation (Pause et al. 1994). Phosphorylation of 4E-BP by mTOR inactivates 4E-BP, and in doing so, allows eIF4E to carry out its function in translation initiation. eIF4E recognizes and binds to the 5′ cap structure of mRNA (Feigenblum and Schneider, 1996), and this complex then associates with two additional initiation factors, eIF2A and eIF4G to form the eIF4F complex (Gingras et al. 1999). This complex has an important role in initiation because it binds the 43S preinitiation complex to the 5′ end of capped mRNA (Pestova et al. 2001).

While no data are available regarding the effect of Akt inhibition on protein synthesis, the constitutively active form of Akt has been shown to increase the rate of protein synthesis (Hajduch et al. 1998; Ueki et al. 1998). In addition, inhibition of PI3K, the up-

stream regulator of Akt, significantly reduces basal protein synthesis (Sanchez-Margalet 1994; Senthil et al. 2002; Freed et al. 2003). Further, inactivation of Akt downstream targets mTOR (Kimball et al. 1998), eIF2B (Oldfield et al. 1994; Hardt et al. 2004), or eIF4E (Kawasome et al. 1998; Senthil et al. 2002) also inhibit protein synthesis. Thus, if calpain activation does in fact impair the signaling of Akt to its downstream targets, this may contribute to the reduction in protein synthesis caused by reduced skeletal muscle use.

The Critical Role of Akt in Skeletal Muscle

Previous research has convincingly demonstrated the important role of Akt in the maintenance of skeletal muscle mass (Bodine et al. 2001b; Cho et al. 2001; Rommel et al. 2001; Pallafacchina et al. 2002; Peng et al. 2003). For example, inhibition of Akt causes atrophy in myotubes (Rommel et al. 2001) and Akt knockout mice display severe skeletal muscle atrophy (Cho et al. 2001; Peng et al. 2003). Further, while reduced skeletal muscle use causes a reduction in Akt activity, a decrease in Akt protein content, and skeletal muscle atrophy (Bodine et al. 2001b; Childs et al. 2003), concomitant treatment of denervated skeletal muscle with an active form of Akt attenuates muscle atrophy (Bodine et al. 2001b). Akt also plays an important role in the hypertrophic response in skeletal muscle. Using a model of skeletal muscle overload known to induce muscle hypertrophy, compensatory hypertrophy, Bodine et al. (2001b) observed significant increases in both Akt content and activation. Moreover, when skeletal muscles subjected to compensatory hypertrophy were simultaneously treated with an inhibitor of an important downstream target of Akt, mTOR, 95% of the hypertrophy was prevented (Bodine et al. 2001b). Hence, the Akt signaling pathway appears to be a critical mediator of the trophic state of skeletal

muscle. Although calpain activation may destabilize the HSP 90/Akt complex, and consequently reduce Akt activation and signaling, this line of research has not been investigated.

In summary, it is widely held that the calpain proteases play only a minor role in reduced-use skeletal muscle atrophy because inhibition of these proteases accounts for only a small fraction of elevated protein degradation following relatively chronic (3-9 days) catabolic treatments while inhibition of the ubiquitin-proteasome system accounts for the large majority of accelerated proteolysis under the same conditions. However, inhibition of calpain following a relatively brief period of reduced use (18 hours) prevents the rise in proteolysis and calpastatin overexpression throughout nine days of reduced muscle use attenuates skeletal muscle atrophy. Recent research indicates that other proteases act upstream of the ubiquitin-proteasome system (Du et al. 2004), and the same may be true for the calpain proteases. Calpain cleavage may serve as a degron for ubiquitin-proteasome degradation of calpain cleaved polypeptide fragments, while inhibition of HSP 90 chaperone function by calpain may cause ubiquitin-proteasome degradation of HSP 90 client proteins. If calpain activation causes activation of the ubiquitin-proteasome system, this may explain why calpain inhibition following relatively chronic catabolic treatments fails to suppress protein degradation. Although it has been suggested that the calpain and ubiquitin-proteasome systems work in sequence, this hypothesis has not been addressed experimentally. Thus, one goal of the proposed investigation will be to determine whether the calpain act upstream of the ubiquitin-proteasome pathway in rat skeletal muscle.

Akt has several downstream targets that function in the translation phase of protein synthesis. Research has shown convincingly the critical role of the Akt signaling pathway in protein synthesis signaling and the maintenance of skeletal muscle mass. The molecular complex formed between the chaperone protein HSP 90 and Akt appears to be necessary for Akt stability and proper function. Calpain cleaves HSP 90 in non-skeletal muscle cells, and calpain activation also destabilizes the chaperone/client protein complex. While calpain activation may inactivate this vital protein synthesis-signaling pathway, no data are currently available on this topic. Therefore, the proposed investigation will also determine whether activation of the calpain proteases inactivates components Akt signaling pathway in rat skeletal muscle.

In non-skeletal muscle cells, destabilization of the HSP 90/client protein complex causes ubiquitin-proteasome degradation of numerous HSP 90 client proteins and also causes inactivation of Akt. Given the critical role of HSP 90/client protein complex in both ubiquitin-proteasome degradation and Akt signaling, elucidating whether calpain activation causes destabilization of the HSP 90/Akt complex in skeletal muscle may offer important insight as to potential mechanisms of protein ubiquitination and/or Akt inactivation caused by calpain activation. Therefore, an additional goal of the proposed investigation will be to determine whether calpain activation disrupts the HSP 90/Akt complex in rat skeletal muscle.

CHAPTER 3
MATERIALS AND METHODS

The University of Florida Institutional Animal Care and Use Committee approved all experimental procedures. Based on data observed in the literature, a statistical power analysis was calculated using an alpha level of 0.05 and a power of 0.8, and the estimated number of animals necessary per group to detect differences between means was calculated to be 11. Female Sprague-Dawley rats weighing 230-250 grams were obtained from Harlan. During a one-week acclimation period, rats were housed in a temperature and humidity controlled room maintained on a 12-hour light/dark cycle, and have free access to a standard rat chow and water.

Model of Skeletal Muscle Calcium Overload

An experimental model of calcium overload was utilized for the present experiments. The *in vitro* model, originally developed to measure total proteolysis in isolated muscle, has been employed by numerous scientists to investigate the calcium overload in rat skeletal muscle (Zeman et al. 1985; Furuno and Goldberg, 1986; Benson et al. 1989; Bhattacharyya et al. 1993; Gissel and Clausen, 2003). All animals were anesthetized to a surgical plane of anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg). Diaphragm muscles were removed and randomly assigned to one of four different groups (Figure 1.): control Krebs physiological solution (CON), physiological solution containing 3.5mM calcium (Ca^{2+}), physiological solution with 3.5mM Ca^{2+} and the calpain inhibitor calpeptin (1μM) (Ca^{2+}/CI), or a physiological solution with 3.5mM Ca^{2+} and the

proteasome inhibitor epoxomicin (30μM) (Ca^{2+}/PI). Since the protease inhibitors were dissolved in DMSO, an equivalent concentration of DMSO was added to the physiological solution of the CON and Ca^{2+} groups (0.02%). Of note, the Ca^{2+}/PI group was not necessary in the second set of experiments. While calpeptin also inhibits the lysosomal proteases, previous investigations indicated that this experimental model of calcium overload does not activate the lysosomal proteases (Zeman et al. 1985; Baracos et al. 1986; Furuno and Goldberg, 1986; Furuno et al. 1990). Diaphragm muscles were carefully removed, cut in half, and fixed to a plexiglass support. The muscles were allowed to shorten since this has been shown to maximally activate Ca^{2+}-mediated protein degradation (Baracos and Goldberg, 1986; Furuno et al. 1990). All muscles were pre-incubated for 30 minutes in a standard Krebs-Ringer incubation medium (pH 7.4) containing: 10 mM glucose, 25 mM sodium bicarbonate, 1.5 mM $CaCl_2$, 0.1 mM isoleucine, 0.17 mM leucine, 0.2 mM valine, 1 milliunit/mL insulin, and 0.5 mM cycloheximide. The pre-incubation solution also contained one of the two protease inhibitors, or an equivalent concentration of DMSO (0.02%) in the case of the CON and Ca^{2+} groups. The incubation solution was saturated with 95% O_2 / 5% CO_2 and maintained at 37° C through out the experimental protocol. Following pre-incubation, the muscles were transferred to a fresh medium as indicated in Figure 1. Following the one-hour muscle incubation, diaphragm muscles were washed in control medium to remove excess inhibitor, blotted dry, weighed, frozen in liquid nitrogen, and stored at -80° C. The incubation medium was then stored at -20° C for subsequent analysis.

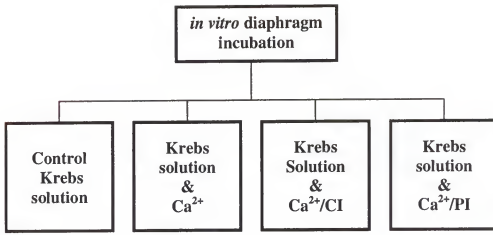


Figure 1. Experimental Design.

Protein Concentration

Protein concentration was determined using the Bradford or BCA method, using a bovine serum albumin standard. All assays will be run in triplicate.

Calpain Cleavage Product

To confirm activation of the calpain proteases, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were employed. Talin is a well-characterized calpain substrate and the appearance of the 190 kDa talin fragment has been used as an indicator of calpain proteolysis (Koh and Tidball, 2000). Therefore, the 190 kDa talin fragment was utilized as a measure of calpain proteolytic activation. Equal amounts of protein (20 μg) from the cytosolic fraction were loaded on a 4-20% gradient gel, separated, and then transferred to a nitrocellulose membrane. The membranes were then blocked for one hour at room temperature with TBS-T blocking buffer (50 mM Tris base, pH 7.4, 150 mM NaCl, 1% Tween 20) and 5% nonfat dry milk. Next, the blots were incubated for overnight at 4°C with a monoclonal mouse anti-talin primary antibody (1:1000, Sigma-Aldrich, St. Louis, MO). The blots were then

washed with TBS-T wash buffer for 3 x 10 minutes at room temperature and subsequently incubated for 1 hour with the secondary antibody (anti-mouse IgG horseradish peroxidase; 1:1000, Amersham, UK). After another 3 x 10 minutes wash, blots were exposed to an enhanced chemiluminescence system (Amersham Pharmica Biotech, UK) for 1 minute and to X-ray film for approximately 10 seconds. The density of the 190 kDa talin fragment was analyzed using Kodak ID Image Analysis Software (Eastman Kodak Scientific Imaging Systems, Rochester, NY). Of note, this general procedure was performed in subsequent SDS-PAGE/Western blot experiments.

Ubiquitin-Conjugated Protein Content

The content of ubiquitin-conjugated proteins was determined in the cytocolic and myofibrillar fractions by SDS-PAGE / Western blot and densitometry analysis. Approximately 15 mg of frozen muscle was homogenized on ice in a glass-on-glass homoginizer (Kontes) in 20 volumes of ice cold buffer (50 mM Tris-HCL, 1 mM EDTA, 1 mM EGTA, 50 mM E64, 2.5 μM pepstatin A, 10 % glycerol, pH 8). Homogenates were centrifuged at 1500 x g for 10 minutes at 4° C to separate the myofibrillar (pellet) fraction. The myofibrillar fraction was washed three times with homogenizing buffer containing 1% Triton X-100, and resuspended in 8M Urea, 50 mM Tris-HCl (pH 7.5) and stored at -80° C for subsequent analysis. The supernatant from the initial 1,500 x g centrifugation was collected and further centrifuged at 100,000 x g for 1 hour at 4° C. The supernatant (cytosolic fraction) was collected and stored at -80° C for subsequent analysis. Protein concentration was determined and standardized. The general SDS-PAGE/Western blotting procedure described above was carried out using a monoclonal anti-ubiquitin pri-

mary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). The content of ubiquitin-conjugated proteins was measured by densitometry.

Proteasome Enzyme Activity

N-Succinyl-Leu-Leu-Val-Tyr 7-amido-4-methylcoumarin (LLVY), N-tert-Boc-Leu-Arg-Arg 7-amido-4-methylcoumarin (LRR), and Cbz-Leu-Leu-Leu-al (MG132) were purchased from Sigma (St. Louis, MO). Cbz-Leu-Leu-Glu 7-amido-4-methylcoumarin (LLE) was purchased from CalBiochem (San Diego, CA) and lactacystin was purchased from Boston Biochem (Cambridge, MA). Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-AMC were used to measure CT-L, T-L, or PGPH proteasome activity, respectively. Proteasome enzymatic activity was determined according to Vigouroux et al. (2003), with slight modifications. Protein concentration was determined and sample protein concentration standardized. Five μg of cytosolic fraction (from above) was incubated with the reaction buffer (50 mM Tris-HCL, 1 mM DTT, pH 8) and 40 μM of substrate ± 100μM proteasome inhibitor or an equivalent volume of DMSO (< 5% of the total volume), for a total assay volume was 50 μl. After 30-minute incubation at 37ºC, the reaction was stopped with 200 μl of ice-cold methanol. Enzyme activity was calculated as the difference in free AMC in the absence or presence of the proteasome inhibitor lactacystin or MG132 (PGPH). The released free AMC was measured fluorometrically at an excitation wavelength of 370 nm and emission wavelength of 430 nm, quantified by a free AMC standard curve, and expressed as nmol/min/mg.

Total Protein Degradation

Total protein degradation can be measured by the release of tyrosine into the incubation medium because skeletal muscle can neither synthesize nor degrade the amino

acid tyrosine (Waalkes and Udenfriend, 1957). Protein degradation was measured as previously described (Yimlamli et al. 2005). The frozen incubation medium was thawed and 0.5 mL of the medium was added to 2 mL dH_2O and 0.5 mL of 30% tricarboxilic acid (TCA). The mixture was then incubated for 10 minutes, followed by a 10 minute centrifuge at 400 x g. 1 mL of the supernatant will be transferred to a tube containing 0.5 mL of 1% nitrosonapthol (w/v) and 0.5 mL of nitric acid reagent (20% nitric acid, 2.5% of NaN_2 (w/v)). The sample was then incubated in a water bath at 55 ° C for 30 minutes. Once the solution cooled for 15 minutes at room temperature, 5 mL of ethylene dichloride was added and the solution, vortexed and then centrifuged for 15 minutes at 2500 x g. The supernatant was collected and the amount of tyrosine was determined fluorometrically at an excitation wavelength of 460 nm and emission wavelength of 570 nm, quantified by a free AMC standard curve, and expressed as nmol/mg/1 hour.

Protein Synthesis Signaling

The Akt/mTOR/GSK-3β signaling pathway has been consistently shown to play a role in protein synthesis (Hajduch et al. 1998; Ueki et al. 1998; Hornberger et al. 2004; Kubica et al. 2005). Because Akt phosphorylation correlates with its kinase activity (Burgering and Coffer, 1995; Zinda et al. 2001; Xu et al. 2003; Zhang et al. 2004; Varma et al. 2005), Akt phoshorylation is frequently used as an indicator of activity (Bodine et al. 2001b; Rommel et al. 2001; Sakamoto et al. 2004). Akt phosphorylation and protein content were determined by Western blot as described by Sakamoto et al. (2004), with slight modification. Approximately 15 mg of muscle were homogenized in 20 volumes of ice cold buffer lysis buffer (Cell Signaling Technology, #9803) containing 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 1

phosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 µg/ml leupeptin, and 1 mM PMSF) and centrifuged at 14,000 x *g* for 10 minutes at 4° C. The supernatant was collected, and protein concentration was determined and standardized. Next, the general SDS-PAGE/Western procedure described above was employed to determine phosphorylation and protein content using antibodies purchased from Cell Signaling Technologies (Beverly, MA) for Akt (#4058 anti-phospho-Akt antibody; #9272 anti-Akt antibody, respectively, 1:1000), mTOR (#2971; anti-phospho-mTOR Ser 2448; #2972 anti-mTOR, respectively, 1:250) and GSK-3β (#2971 anti-phospho-GSK-3β Ser 9; anti-GSK-3β; #9332, respectively, 1:1000). Protein phosphorylation and content were measured by densitometry.

HSP 90/Akt Binding

To determine the amount of HSP 90 associated with Akt, Akt was immunoprecipitated using Cell Signaling Technology's anti-Akt antibody (# 9272). Immunoprecipitation was conducted according to manufacturer's instructions, with slight modification. Fifteen milligrams of diaphragm muscle was homogenized (1:20 w/v) in lysis buffer (Cell Signaling Technology, #9803) containing 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 µg/ml leupeptin, and 1 mM PMSF and centrifuged 10 minutes, at 4°C, at 1,000 x *g*. The supernatant (cell lysate) was collected, and protein concentration was determined using the Pierce BCA protein assay (Pierce, Rockford, IL) and normalized. The primary antibody was diluted ten-fold and 20 µl of the antibody was added to 700 µg of the cell lysate and incubated overnight at 4°C with gentle rock. Next, 20µl of Protein A Agrose beads from Santa Cruz Biotechnology (Santa Cruz, CA, # sc-

2003) was added and gently rocked overnight. The lysates were then centrifuged at 1,000 x *g*, for 5 minutes at 4°C. The supernatant was discarded and the pelleted immunoprecipitates were washed three times by adding 500 µl of lysis buffer and gently rotating the lysates 10 times using a rotary incubator, each time repeating the centrifugation step above. After the final wash, the supernatant was discarded and the pellet was resuspended in 50 µl 1X SDS sample buffer. Samples were boiled for 10 minutes, and subsequently centrifuged for 5 minutes at 1,000 x *g* at room temperature. 15 µl of the supernatant was loaded on a 4-20% gel, resolved by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblot analysis. While the two HSP 90 isoforms, HSP 90*a* and HSP 90*B*, are 86% homologous, the isoforms may have different functions (Moore et al. 1989). Since no data are available regarding HSP 90/Akt binding in skeletal muscle, we investigated the effect of calpain activation on the binding of both HSP 90 isoforms to Akt. The general Western blot procedure described above was followed using anti- HSP 90*a* (# PA3-013) or anti- HSP 90*B* (# PA3-012) antibodies (1:500 dilution) purchased from Affinity Bioreagents (Golden, CO). The amount of HSP 90 bound to Akt was measured by densitometry.

HSP 90 Content

Calpain activation reduces HSP 90 content in endothelial cells (Su and Block, 2000) and a reduction in protein content could limit the amount of HSP 90 available to chaperone Akt. To gain insight into potential mechanisms of reduced HSP 90/Akt binding, we also determined the effect of calpain activation on HSP 90*a* and HSP 90*B* content (Affinity Bioreagents, PA3-013; PA3-012, respectively, 1:500 dilution), using the super-

natant from the Akt Western protocol. HSP 90α and HSP 90β content were measured by densitometry.

Statistics

Data were analyzed using a one-way analysis of variance (ANOVA). Where indicated, Tukey's multiple comparison test was used to assess differences among the means. The level of significance will be set at $P<0.05$. Values reported are means ± S.E.M.

CHAPTER 4
RESULTS

Effect of Muscle Incubation on Calpain Activation

Since calpain cleaves intact talin to a 190 kDa fragment, calpain activity was determined by measuring the amount of 190 kDa talin fragment. Incubation of diaphragm muscle strips with calcium (Ca^{2+}) alone increased the level of the 190 kDa talin fragment by about 135% (Fig 2). Addition of the calpain inhibitor, calpeptin, to the Ca^{2+} treatment (Ca^{2+}/CI) prevented the calcium-mediated rise in fragmented talin, indicating that calpain was responsible for the appearance of the talin fragment. There was also a significant increase in cleaved talin in the Ca^{2+}/proteasome inhibitor (Ca^{2+}/PI) group compared to control (CON) and Ca^{2+}/CI (115% and 150%, respectively). Further, the amount of cleaved talin was similar in the Ca^{2+} and Ca^{2+}/PI groups, suggesting that the proteasome inhibitor did not affect calpain activation.

Effect of Calpain Activation on Ubiquitin-Conjugated Protein Content

The attachment of ubiquitin to protein substrates serves as a proteasomal degradation signal; in fact, most of the proteins degraded by the proteasome are ubiquitin conjugated. Ca^{2+} treatment in combination with the proteasome inhibitor (Ca^{2+}/PI) significantly increased the content of cytosolic ubiquitin-conjugated proteins (approximately 170%) compared to all other groups. However, neither Ca^{2+}or Ca^{2+}/CI affected the level of cytosolic ubiquitin-conjugates (Figure 3A). Figure 4A shows the content of myofibrillar

ubiquitin-conjugates. There were no significant differences in myofibrillar ubiquitin-conjugated protein content among the groups.

Effect of Calpain Activation on Proteasome Enzyme Activity

If the calpain proteolytic pathway acts upstream of the ubiquitin-proteasome pathway, calpain activation would be expected to increase proteasome activity. While the proteasome inhibitor epoxomicin significantly reduced CT-L, T-L, and PGPH enzyme activity (99%, 85%, 95%), incubation with Ca^{2+} or Ca^{2+}/CI did not affected proteasome enzymatic activity (Table 1).

Effect of Calpain Activation on Total Protein Degradation

Although no changes in proteasome enzymatic activity were detected in the current investigation, this does not rule out the possibility of ubiquitin-proteasome activation at an earlier time point. To address this question, we incubated skeletal muscles with Ca^{2+} and the proteasome inhibitor epoxomicin. If calpain acts upstream of the ubiquitin-proteasome system, then calpain activation with concomitant proteasome inhibition should prevent the calpain-mediated rise in proteolysis. As shown in Figure 5, total protein degradation was significantly higher in the Ca^{2+} group compared to CON (84%), and this effect was prevented by either calpain inhibition or proteasome inhibition. In fact, acute calpain activation increased proteasome dependent protein degradation by 45%. There was no difference in proteolysis between Ca^{2+}/CI and Ca^{2+}/PI groups (Figure 5). Of note, the protease inhibitors appeared to act specifically, since the proteasome inhibitor epoxomicin had no effect on calpain activity (Figure 2) and the calpain inhibitor calpeptin had no effect of proteasome enzyme activity (Table 1). Taken together, these

data demonstrate that proteasome enzyme activity is necessary for the calpain-mediated rise in proteolysis, and thus support the hypothesis of sequential proteolytic pathways.

Effect of Calpain Activation on Protein Synthesis Signaling

Effect of Calpain Activation on Akt

Because calpain cleaves a key Akt regulator protein, HSP 90, we theorized that calpain activation would adversely affect Akt and its downstream targets mTOR and GSK-3β. Of note, the Ca^{2+}/PI was group was not necessary for the protein synthesis signaling experiments, and therefore excluded. As shown in Figure 6A, treatment of muscle strips with Ca^{2+} significantly reduced Akt phosphorylation (by 35%), an indicator of Akt activity. The reduction in Akt phosphorylation was prevented in muscles concomitantly treated with Ca^{2+} and the calpain inhibitor, indicating that the Ca^{2+} induced Akt inhibition was calpain mediated. While Ca^{2+} tended to reduce Akt content, there were no significant differences in Akt content between the groups (Figure 6B).

Effect of Calpain Activation on mTOR

Mammalian target of rapamycin (mTOR) phosphorylation was reduced by approximately 50% following Ca^{2+} incubation and calpain inhibition abolished the Ca^{2+} mediated reduction in mTOR phosphorylation (Figure 7A). Similar results were obtained for mTOR protein content, in that, Ca^{2+} treatment reduced the level of mTOR by about 50% and calpain inhibition prevented this reduction (Figure 7B).

Effect of Calpain Activation on GSK-3β

Akt promotes protein synthesis by phosphorylating (inactivating) GSK-3β, a negative regulator of protein synthesis. GSK-3β phosphorylation was reduced (activated) by about 40% with the Ca^{2+} incubation (Figure 8A), and calpain inhibition blocked this

GSK-3β activation. Although incubation with Ca^{2+} resulted in a trend towards reduced GSK-3β content, the level of GSK-3β did not differ between groups (Figure 8B).

Effect of Calpain Activation on Akt /HSP 90 Binding

Inhibition of HSP 90 has been demonstrated to reduce the binding of HSP 90 to its client proteins. Since calpain cleaves HSP 90 and causes reductions in HSP 90 content, it stands to reason that calpain activation may also reduce the association of HSP 90 and Akt. To determine whether calpain activation affected the protein-protein interactions between Akt and HSP 90, Akt was immunoprecipitated and subjected to SDS-PAGE. The protein on the gel was then electrophoretically transferred to a nitrocellulose membrane and the membrane immunoblotted with anti-HSP90 antibody. Experiments to determine the levels of HSP 90α and HSP 90β bound to Akt were run concurrently, using the same cell lysates. HSP 90β was found to be associated with Akt, whereas HSP90α was not (data not shown). Calpain activation did not affect the amount of HSP 90β associated with Akt (Figure 9).

Effect of Calpain Activation on HSP 90 Content

Since a reduction in HSP 90 content could contribute to reductions in HSP 90/Akt binding, we also determined the effect of calpain activation on the amount of HSP 90. While calpain activation had no effect on the level of HSP 90α (Figure 10A), Ca^{2+} incubation significantly diminished HSP 90β content (by 33%; Figure 10B) and the Ca^{2+} mediated reduction in HSP 90β was prevented by calpain inhibition.

Confirmation of Proper SDS-PAGE Protein Loading and Electrophoretic Transfer

To confirm that the total amount of protein on the nitrocellulose membrane did not differ between groups, membranes were stained with Panceau S staining solution.

Figure 11A shows an example of a Panceau S stained Western blot membrane. There were no differences in total protein (Figure 11B), indicating equivalent protein loading during SDS-PAGE and proper electrophoretic transfer of proteins to the membrane. This indicates that alterations in phosphorylation and protein content are likely due to calpain activation rather erroneous protein loading and/or protein transfer.

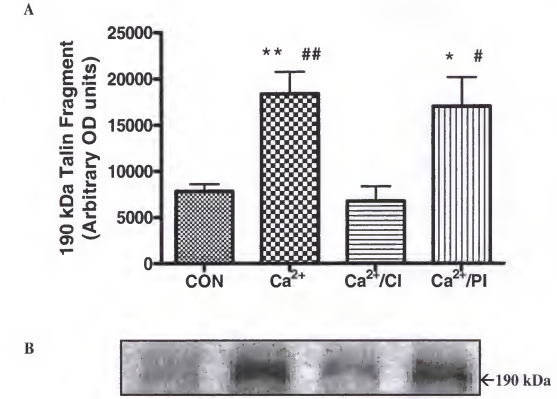
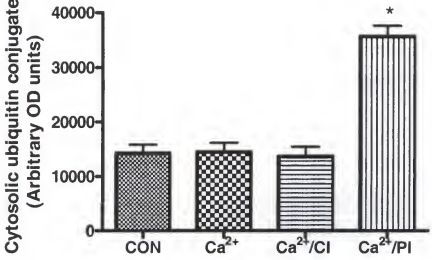


Figure 2. Effect of muscle incubation on proteolytic cleavage of talin. A) Densitometric analysis of cleaved talin in control (CON), calcium (Ca^{2+}), calcium + calpain inhibitor (Ca^{2+}/CI), and calcium + proteasome inhibitor (Ca^{2+}/PI) treated muscles. B) Representative Western blot of 190 kDa talin fragment. Results shown are mean values ± SEM. ** Significantly different from CON ($P < 0.01$); * significantly different from CON ($P < 0.05$); ## significantly different from Ca^{2+}/CI ($P < 0.01$); # significantly different from Ca^{2+}/CI ($P < 0.05$).

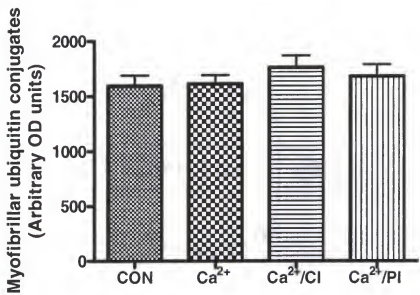
A



B

Figure 3. Effect of calpain activation on cytosolic ubiquitin-conjugated protein content. A) Densitometric analysis; and B) Representative Western blot of cytosolic ubiquitin conjugates. Results shown are mean values ± SEM. * Significantly different from all other groups ($P < 0.001$).

A



B

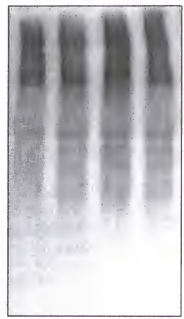


Figure 4. Effect of calpain activation on myofibrillar ubiquitin-conjugated protein content. A) Densitometric analysis; and B) Representative Western blot of myofibrillar ubiquitin conjugates. Results shown are mean values ± SEM.

Table 1. Effect of calpain activation proteasome enzyme activity.

	CT-L	T-L	PGPH
CON	0.1653 ± 0.008	0.02007 ± 0.002	0.07227 ± 0.007
Ca^{2+}	0.1702 ± 0.013	0.01938 ± 0.002	0.06922 ± 0.006
Ca^{2+}/CI	0.1680 ± 0.013	0.01926 ± 0.003	0.07086 ± 0.004
Ca^{2+}/PI	0.00025 ± 0.00005 *	0.0034 ± 0.002 *	0.0040 ± 0.003 *

Values presented are means ± SEM. Activities are expressed in nmol/mg/min.
*Significantly different from all other groups ($P < 0.001$).

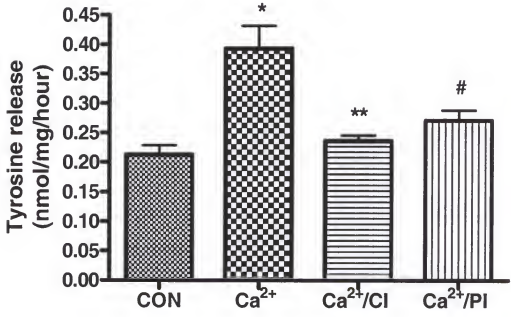


Figure 5. Effect of calpain activation on total protein degradation. Results shown are mean values ± SEM. * Significantly different from CON ($P < 0.001$); ** significantly different from Ca^{2+} ($P < 0.001$); # significantly different from Ca^{2+} ($P < 0.01$).

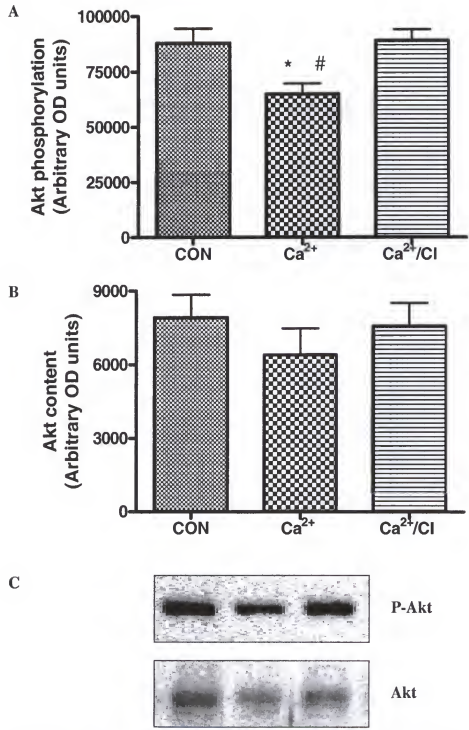


Figure 6. Effect of calpain activation on Akt. Densitometric analysis of A) Akt phosphorylation; B) Akt content; C) Representative Western blot of Akt phosphorylation and Akt content. Results shown are mean values ± SEM. * Significantly different from CON ($P < 0.05$); # significantly different from Ca^{2+}/CI ($P < 0.05$).

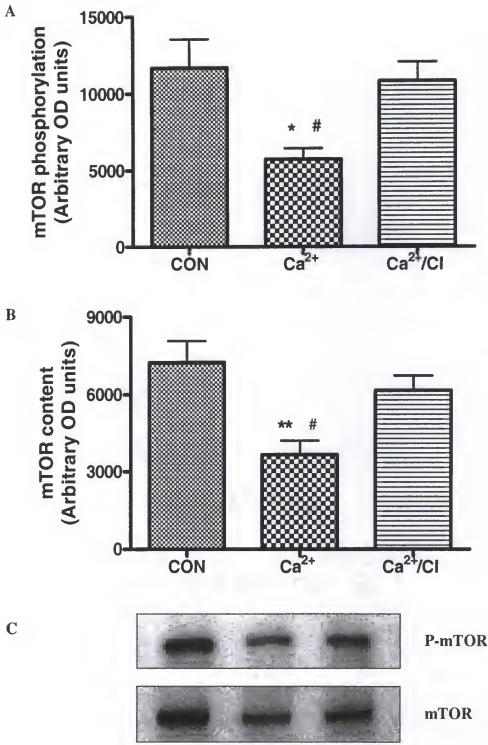


Figure 7. Effect of calpain activation on mTOR. Densitometric analysis of A) mTOR phosphorylation; B) mTOR content; C) Representative Western blot of mTOR phosphorylation and mTOR content. Results shown are mean values ± SEM. * Significantly different from CON ($P < 0.05$); ** significantly different from CON ($P < 0.01$); # significantly different from Ca^{2+}/CI ($P < 0.05$).

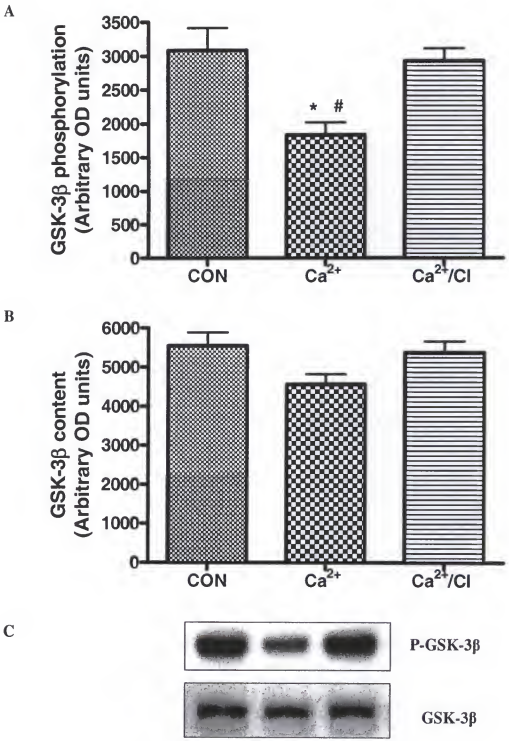


Figure 8. Effect of calpain activation on GSK-3β. Densitometric analysis of A) GSK-3β phosphorylation; B) GSK-3β content; C) Representative Western blot of GSK-3β phosphorylation and GSK-3β content. Results shown are mean values ± SEM. * Significantly different from CON ($P < 0.01$); # significantly different from Ca^{2+}/CI ($P < 0.05$).

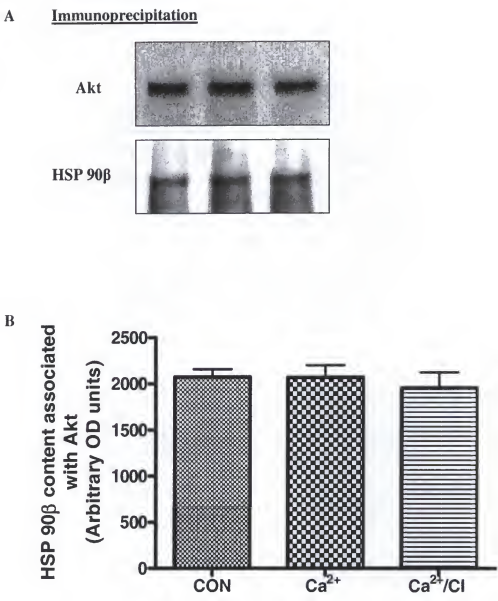


Figure 9. Effect of calpain activation on Akt/HSP 90β binding. A) Representative Western blot of immunoprecipitated Akt and HSP 90β; B) densitometric analysis of HSP 90β coprecipitated with Akt. Results shown are mean values ± SEM.

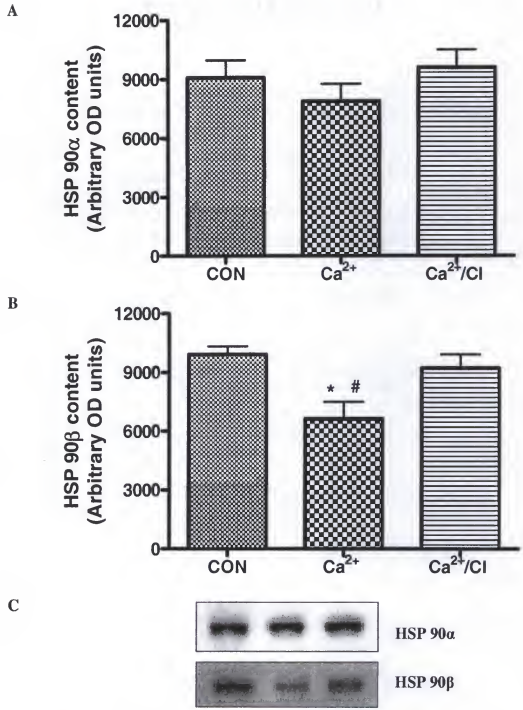


Figure 10. Effect of calpain activation on HSP 90 content. Densitometric analysis of HSP 90α content (A); and HSP 90β content (B); C) Representative Western blot of HSP 90α and HSP 90β content. Results shown are mean values ± SEM. * Significantly different from CON ($P < 0.05$); # significantly different from Ca^{2+}/CI ($P < 0.05$).

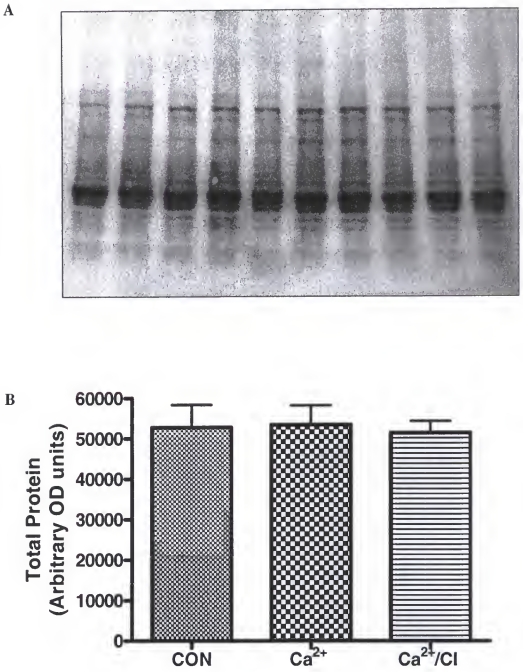


Figure 11. Confirmation of proper protein loading and transfer during SDS-PAGE and electrophoretic transfer. A) Representative Panceau S stained membrane; B) densitometric analysis of total protein from the Panceau S stained membrane shown in 11A. Results shown are mean values ± SEM.

CHAPTER 5
DISCUSSION

Effect of Calpain Activation on Total Proteolysis and Proteasome Activity

Determining the mechanism(s) of protein loss during catabolic conditions is important to developing interventions to prevent muscle wasting. While the ubiquitin-proteasome pathway has been shown to degrade a large fraction of proteins in atrophying skeletal muscle (Taillandier et al. 1996), some data suggest that proteases acting upstream of the ubiquitin-proteasome pathway initiate the degradative process. For example, Du et al. (2004) recently reported that caspase-3 increased ubiquitin-proteasome pathway mediated proteolysis. One major finding of the present study was that the proteasome is necessary for calpain-mediated protein degradation, indicating that the calpain proteases also act upstream of the proteasome system. The data further demonstrate that brief calpain activation is sufficient to cause a large (45%) increase in proteasome-mediated protein degradation. Our finding is consistent with a recent investigation linking the two pathways, which found that calpain activation increased proteasome activity in cultured myotubes (Menconi et al. 2004). Our data have important implications because it lends further support for the notion that calpain is an initial step in catabolic conditions, and therefore offers a practical target for the development of interventions to prevent muscle wasting.

The unchanged proteasome enzyme activity in the present investigation was surprising given that earlier studies have shown elevated proteasome activity with Ca^{2+}

overload (Kawahara and Yokosawa; 1994; Aizawa et al. 1996; Menconi et al. 2004). The reduction in HSP 90β may explain why there were no apparent changes in proteasome activity at the end of the muscle incubation. Recent research indicates that HSP 90 plays key roles in assembling the 26S proteasome and maintaining the 26S complex (Imai et al. 2003). Initially, there may have been a sufficient amount of HSP 90β to assemble and maintain the 26S proteasome, and thus support early increases in proteasomal degradation. However, the continued calpain activation and subsequent reductions in HSP 90β content may have resulted in 26S disassembly at later time points, thereby repressing proteasome activity. Alternatively, calcium overload can cause oxidative stress (Johnson et al. 1988; Wilkinson and Jacob, 2003) and HSP 90 has been shown to protect the proteasome from oxidative inhibition (Conconi et al. 1998). If our model of Ca^{2+} overload caused oxidative stress, it may be that HSP 90 protected the proteasome at the outset; however, the subsequent loss of HSP 90β combined with oxidative stress may have restrained proteasome activity towards the end. The idea of a temporal increase in proteasome activity is supported by previous studies in which Ca^{2+}-mediated proteasome activation was found to be transient (Kawahara and Yokosawa, 1994; Aizawa et al. 1996). It therefore seems plausible that proteasome activity was elevated at an earlier time point than studied here. The finding that proteasome inhibition through out the entire treatment period prevented calpain-mediated protein degradation supports this notion.

Effect of Calpain Activation on Ubiquitin-Conjugates

Ubiquitin-conjugation marks proteins for degradation by the 26S proteasome, and ubiquitination is thought to be the rate-limiting step in the ubiquitin-proteasome system (Solomon et al. 1998b). Because calpain cleavage may cause protein modifications lead-

ing to ubiquitin-conjugation, we examined whether calpain activation altered the level of ubiquitinated proteins. We found that acute calpain activation did not affect the amount of ubiquitin-conjugated proteins in the cytosolic or myofibrillar fraction. A possible explanation is that calpain cleaved proteins are degraded by the 26S proteasome independent of ubiquitin-conjugation. This explanation, however, seems unlikely since only a limited number of proteins are degraded by the 26S proteasome in an ubiquitin-independent manner (see Orlowski and Wilk, 2003 for review). Alternatively, the 20S proteasome, which catalyzes ubiquitin-independent proteasome proteolysis (Shringarpure et al. 2003), may degrade calpain cleaved protein fragments. This also seems doubtful since current knowledge of the function of the 20S proteasome indicates that the 20S preferentially degrades oxidatively damaged proteins (Shingarpure et al. 2003). In addition, the ubiquitin system has been shown previously to catalyze a major fraction of the protein degradation in normal and atrophying skeletal muscle (Solomon et al. 1998b; Solomon et al. 1998a). Given the predominant role of the ubiquitin system in protein degradation, it seems unlikely that the large, proteasome-mediated rise in proteolysis observed in the current investigation occurred independent of the ubiquitin system.

Although the possibility of ubiquitin-independent 26S proteolysis and/or activation of the 20S proteasome cannot be ruled out, it seems likely that calpain cleaved polypeptide fragments may have been ubiquitin-conjugated and rapidly degraded by the 26S proteasome. Calpain cleavage may expose amino acid residues that are known to increase the propensity of ubiquitin-conjugation by the N-end rule pathway, the predominant ubiquitin-conjugation pathway in skeletal muscle. The presence of acidic, basic, or bulky hydrophobic N-terminal amino acid residues leads to rapid ubiquitination and protea-

somal degradation (Bachmair et al. 1986; Gonda et al. 1989; Richter-Ruoff et al. 1992) and previous research indicates that calpain cleaves proteins at amino acids fitting this description (Sasaki et al. 1984). Further, Nelson and Traub (1983) demonstrated previously that calpain cleaved proteins at the N-terminus. Thus, there is strong circumstantial evidence supporting the idea that calpain cleaved polypeptide fragments may become ubiquitin-conjugated, although this has yet to be confirmed experimentally.

Potentially Novel Role for Calpain in Activating the Ubiquitin System

Our findings suggest that calpain activation may also modulate gene expression of the ubiquitin system. Two muscle specific, E3 ubiquitin ligases have been identified as part of the so called "atrophy program", a set of transcriptional adaptations common to various types of muscle atrophy (Gomes et al. 2001; Bodine et al 2001a). Genetic knockout of atrogin-1 (also known as MAFbx) or muscle ring finger 1 (MuRF1) attenuates skeletal muscle atrophy in mice, likely by preventing the ubiquitin-conjugation and subsequent degradation of muscle proteins (Bodine et al. 2001a). The forkhead box O (FOXO) family of transcription factors regulates atrogin-1 and MuRF1 expression, and there is now strong evidence that Akt is a negative regulator of the FOXO transcription factors (Brunet et al. 1999; Sandri et al. 2004; Stitt et al. 2004). For instance, phosphorylated (activated) Akt phosphorylates FOXO1, and prevents its translocation to the nucleus (Brunet et al. 1999). Our finding that calpain activation reduces Akt phosphorylation suggests that calpain may promote the transcription of two ubiquitin ligases known to play significant roles in skeletal muscle atrophy. This finding introduces a novel mechanism by which calpain may mediate activation of the ubiquitin-proteasome pathway.

Implications for the Role of Calpain in Elevated Protein Degradation

The observation that calpain activation increases proteasome dependent protein degradation raises the question of whether the contribution of calpain to catabolic conditions was previously underestimated. Earlier studies indicated that calpain plays a minor role in elevated proteolysis during catabolic conditions such as reduced muscle use, muscle denervation, starvation, cancer cachexia, sepsis, and metabolic acidosis (Medina et al. 1991; Tiao et al. 1994; Mitch et al. 1994; Baracos et al. 1995; Taillandier et al. 1996). However, in many of these earlier investigations, catabolic insults ranging from three to nine days preceded the removal and incubation of muscles with protease inhibitors to delineate the contribution of the various proteolytic systems to total protein degradation. Many catabolic conditions cause a build-up in ubiquitin-conjugated proteins, substrates for the 26S proteasome, and this increase can be observed in as little as 2 hours (Chai et al. 2001). Our findings, (and the data of Menconi et al. 2004) that calpain acts upstream of the proteasome system, raises the possibility that the accumulation in ubiquitin-conjugated proteins may have masked the contribution of calpain to total proteolysis in previous investigations. In that, calpain inhibition would likely have little immediate impact on proteasome activity, and thus total proteolysis, under conditions in which there is a large surplus of proteasome substrates. Our finding that acute calpain activation in the absence of excess ubiquitin-conjugates caused a large (45%) increase in proteasome-mediated proteolysis supports this idea. This may be why previous investigations have found that calpain inhibition following a catabolic insult fails to reduce elevated proteolysis. In addition, our novel finding that calpain inhibits Akt suggests that calpain may modulate the production of atrogin-1 and MuRF1. If calpain activation does promote

atrogin-1 and MuRF1 production, then this aspect of calpains contribution to elevated proteolysis would also go undetected in the model described above. It therefore seems reasonable to speculate that the contribution of calpain to protein degradation may have been underestimated in those investigations in which calpain activation was unrestrained for the majority of the catabolic insult, and inhibited only during the two-hour muscle incubation in which total protein degradation was measured.

HSP 90β Reductions with Calpain Activation

The finding that calpain diminished HSP 90β content suggests that this isoform is preferentially cleaved by calpain in skeletal muscle. Further, the 30% reduction in HSP 90β content might subsequently reduce cellular HSP 90 chaperone function. However, interpreting the significance of reduced HSP 90β content is complicated since there are no data available regarding the relative abundance of HSP 90α and HSP 90β in skeletal muscle. In fibroblast, the basal level of HSP 90β is approximately 2.5 fold greater that of HSP 90α (Ullrich et al. 1989). A similar proportion has been reported in oncogenic cells, although the level of each isoform is 2-3 folds higher in cancer cells (Ullrich et al. 1989). On the other hand, the mRNA expression of the HSP 90 isoforms is similar in peripheral blood mononuclear cells of healthy humans (Tong and Luo, 2000). Although not compared directly, inspection of HSP 90α and HSP 90β Western blots from the present experiments, which were carried out simultaneously, using the same cell lysates and assay procedures, suggests that the content of HSP 90β is approximately twice that of HSP 90α in skeletal muscle (data not shown). This indicates a principal role for the β isoform in skeletal muscle, and further suggests that calpain activation may well have a large impact on cellular HSP 90 function. Future investigations will be necessary to determine the

proportion and functions of the HSP 90 isoforms in skeletal muscle. Recent oncogenic research using specific HSP 90 inhibitors may offer insight as to potentially important HSP 90 client proteins in skeletal muscle. Given the various roles of chaperone proteins, the finding that calpain activation reduces HSP 90β content may offer new insight regarding the mechanisms of calpain-mediated skeletal muscle atrophy.

Effect of Calpain Activation on the HSP 90/Akt Complex

HSP 90 plays a key role in maintaining the stability and activity of Akt, a protein involved in a variety of cellular functions, including signal transduction, protein synthesis, pro-apoptotic inhibition, and protein degradation. Since calpain activation was shown previously to reduce HSP 90/eNOS binding in mesenteric tissue (Stalker et al. 2003), we hypothesized that calpain activation would reduce HSP 90/Akt binding in skeletal muscle. Coprecipitation experiments revealed that no HSP 90α was associated with Akt. As mentioned above, there appeared to be about twice the level of HSP 90β compared to the α isoform. Therefore, a very large amount of protein may be required to detect the HSP 90α form. While this observation does not rule out the possibility that Akt is a HSP 90α client, it does suggest a minor role for HSP 90α as an Akt chaperone in skeletal muscle. HSP 90β was detected in the immunoprecipitates, however there were no differences in the association of HSP 90β and Akt between the groups. That calpain activation does not reduce HSP 90β/Akt binding suggests that calpain-mediated Akt inhibition occurs independent of HSP 90β. On the other hand, perhaps calpain fractured the HSP 90β/Akt complex, but free HSP 90β rapidly reestablished the complex. Given the vital role of Akt in cell survival, it is conceivable that additional HSP 90β molecules would be enlisted to maintain Akt function. In fact, this may explain why we observed a reduction in Akt ac-

tivity, with no differences in HSP 90β/Akt binding or Akt content. Based on our data, calpain-mediated inhibition of Akt appears to be unrelated to the binding of the two proteins, although the possibility of calpain cleavage followed by rapid re-establishment of the chaperone/client complex cannot be ruled out.

Effect of Calpain Activation on Protein Synthesis Signaling

To our knowledge, this is the first study to demonstrate that calpain inhibits Akt in skeletal muscle. Akt has numerous cellular functions, and thus, the consequences of calpain-mediated Akt inhibition will require further research. However, since the Akt/mTOR/GSK-3β signaling pathway has been consistently linked to protein synthesis (Hajduch et al. 1998; Ueki et al. 1998; Hornberger et al. 2004; Kubica et al. 2004), the observation that calpain adversely affects this signaling network effectively implicates calpain in reduced protein synthesis in skeletal muscle. While the mechanism(s) of calpain-mediated alterations in Akt, mTOR, and GSK-3β signaling cannot be determined from the current experiments, several inferences can be made. The unchanged Akt and GSK-3β content may indicate that calpain inhibited an upstream effector of Akt, and subsequently inactivated the two proteins. However, there were trends towards content reductions for both proteins, and thus, the possibility that these proteins are calpain substrates cannot be ruled out. mTOR inhibition was likely due to the reduction in protein content, since the reductions in content and phosphorylation were similar. Further, it seems likely that mTOR is a preferred calpain substrate given the large (50%) reduction mTOR content. These findings could have broad implications since calpain is activated in a number of catabolic states in which muscle protein synthesis is suppressed, such as re-

duced-use, (Taillandier et al. 1996), sepsis (Wei et al. 2005), cancer cachexia (Busquets et al. 2002), and starvation (Tolnai S and Von Althen, 1987).

In summary, this study demonstrates that calpain acts upstream of the proteasome system, and further shows that acute calpain activation causes a large (45%) increase in proteasome-mediated protein degradation. In addition, our novel finding that calpain inhibits Akt implicates calpain in activation of the ubiquitin system, since there is now strong evidence that Akt regulates the gene expression atrogin-1 and MuRF1, two E3 ubiquitin ligases. The finding that calpain adversely affects the Akt/mTOR/GSK-3β signaling pathway, a signaling pathway consistently linked to protein synthesis, strongly implicates calpain in reduced protein synthesis in skeletal muscle. Finally, HSP 90 appears to have a multitude of chaperone functions (see Csermely et al. 1998 for review). That calpain reduces the level of HSP 90β suggests that calpain activation could adversely affect a variety of cellular functions. Our data offer new insight regarding the mechanisms of calpain-mediated skeletal muscle atrophy and suggest that calpain may play a central role in muscle wasting.

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Ira earned his Bachelor of Science degree from George Mason University, in Fairfax, Virginia, and a Master of Science degree from James Madison University, in Harrisonburg, Virginia. As a University of Florida graduate student, Ira earned a National Aeronautics and Space Administration (NASA) Graduate Student Research Fellowship to support his dissertation work. While working on his dissertation, Ira earned a Postdoctoral Research Fellowship at Duke University and will begin postdoctoral work upon completion of his Ph.D. at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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